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# Antibodies

## A LABORATORY MANUAL

Ed Harlow

Cold Spring Harbor Laboratory

David Lane

Imperial Cancer Research Fund Laboratories



Cold Spring Harbor Laboratory  
1988

## Antibodies A LABORATORY MANUAL

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## *Designing the Peptide*

Probably the most frequently asked question concerning synthetic peptides is what sequence should be used for the immunogen (reviewed in Doolittle 1986). Although there is no one correct answer, enough anti-peptide antibodies have been raised to make suggestions for peptide choices. However, preparing anti-peptide antibodies is still an empirical exercise. What works well for one immunogen may fail completely for another.

## *Choosing the Appropriate Peptide Sequence*

With careful synthesis, coupling, and immunizations, most sequences can be used to induce antibodies specific for the peptide itself. When considering which sequence to use, most people actually want to know how likely will it be that the anti-peptide antibodies will recognize the native protein. Early work suggested that peptides containing hydrophilic amino acids (Hopp and Woods 1981, 1983; Kyte and Doolittle 1982) and proline residues were more likely to be exposed on the surface of the native protein than other sequences, and many peptides have been prepared using these criteria. In assessing the value of these criteria, hydrophilicity is required but is not sufficient to predict the surface location of a particular sequence. Many strongly hydrophilic amino acid sequences are buried in water pockets or form inter- or intramolecular bonds and are thus excluded from interactions with anti-native antibodies. Therefore, hydrophilicity can be thought of as required but not sufficient for choosing peptide sequences (see p. 661 for hydrophilicity values). Hydrophilic peptides are also more likely to be soluble for coupling reactions.

The presence of proline residues in synthetic peptides originally was suggested because  $\beta$ -turns often form portions of known epitopes. However, the presence of proline residues in peptides does not have much predictive value when antisera are tested for binding to the surface of native proteins. Although many excellent anti-peptide antisera have been prepared against sequences with proline residues, there is not sufficient evidence to target prolines when designing peptides.

More recently, several workers have noted that carboxy-terminal sequences often are exposed and can be targeted for anti-peptide sequences. Although using carboxy-terminal sequences does not guarantee that the resulting antibodies will recognize the native protein, a surprisingly high percentage will. Similarly, many amino-terminal regions are exposed, and these also may make good targets.

Another potentially useful parameter for selecting peptide sequences is the "mobility" of the amino acid residues. Originally, it was noted that the regions of a protein that become epitopes often have a higher temperature than other regions, as determined by NMR and X-ray structure (Moore and Williams 1980; Robinson et al. 1983; Tainer et al. 1984; Westhof et al. 1984). Higher temperature in crystallography and NMR distinguishes regions that are more mobile from

regions that are more static. These observations have led to the suggestion that stretches of amino acids that are more flexible are more likely to be epitopes. In the preparation of anti-peptide antibodies, when a peptide is coupled to a carrier molecule, it has a different local environment than in the original protein. When choosing a sequence for antibody production, a region of the protein that is more flexible will be more likely over time to form a structure that is similar to the peptide-carrier conjugate. Although the measure of mobility may become a useful criterion for selecting good peptide sequences, it has not been tested in enough detail to determine whether it will have any predictive value.

At present, a reasonable order of suggestions for choosing peptide sequences would be:

1. If possible, use more than one peptide.
2. Use the carboxyl-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
3. Use the amino-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
4. Use internal hydrophilic regions; perhaps using longer peptides.

### *Size of the Peptide*

The smallest synthetic peptides that will consistently elicit antibodies that bind to the original protein are 6 residues in length. Responses to smaller peptides are typically weak or will not recognize the protein of interest, either in a native or denatured state. Since epitopes consisting of smaller regions have been reported, the lower limit presumably reflects the difficulty of recognizing the smaller peptides coupled to carriers. With peptides of 6 amino acids or slightly larger, the responses vary. Some will generate good antibodies and some will not. Generally, peptides of approximately 10 residues should be used as a lower limit for coupling.

In the literature two strategies are suggested for peptide length. One school suggests using long peptides (up to 40 amino acids long) to increase the number of possible epitopes, while other authors argue that smaller peptides are adequate and their use ensures that the site-specific character of anti-peptide antibodies is retained. Both strategies have been used successfully. Two important preliminary questions to consider are: (1) Does the anti-peptide serum need to recognize the native protein? If so, use longer peptides or prepare anti-peptide antisera against multiple peptides. (2) How good is your peptide synthesis facility? Peptides over 20 residues in length are increasingly difficult to synthesize, yielding products with inappropriate side reactions. Longer peptides also are more likely to contain residues that make the coupling to carrier molecules more difficult. The correct decision between peptides with 10-15 residues and longer peptides will depend on the experimental design and will normally be a compromise between these factors. The safest choice, but also the most expensive, will be to prepare multiple small peptides of 10-15 amino acids in length from various regions of the sequence.

### *Coupling Strategy*

When choosing the sequence for a synthetic peptide, one factor that often is overlooked is the method of coupling. Most coupling methods rely on the presence of free amino, sulfhydryl, phenolic, or carboxylic acid groups. Free amino groups used for coupling will be found on lysine side chains or on the amino-terminal residue. Sulfhydryl groups are found on cysteine side chains, phenolic groups on tyrosines, and carboxylic acid groups on aspartic acids, glutamic acids, and the carboxy-terminal residue. Coupling methods should be used that link the peptide to the carrier via either the carboxy- or amino-terminal residue. When preparing antibodies against the carboxy-terminal region of the protein, the coupling should be done through the amino terminus of the peptide. Similarly, the coupling for amino terminal fragments should be done through the carboxy-terminal region of the peptide. For internal fragments, the major consideration is that the peptide be coupled by an end and not through a central residue.

The easiest strategy to manipulate the type of coupling is to add an extra amino acid on either the amino or carboxyl terminus to allow simple, one-site coupling to the carrier. Any coupling method that potentially can bind to an internal residue should be avoided. Similarly, coupling methods should be chosen that will bind to only one amino acid, if possible. If multiple coupling sites are possible, they should be localized to either the amino or carboxyl terminus, and the coupling should be adjusted to link only through one site per peptide on average. It is important to remember that it is often easier to use different peptides than design elaborate coupling schemes.

### *Choosing the Appropriate Carrier*

Many different carrier proteins can be used for coupling with synthetic peptides. The two most commonly used are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). Both work well in most cases, but each has disadvantages. Because of its large size, KLH is more likely to precipitate during cross-linking, and this can make handling KLH difficult in some cases. On the other hand, BSA is very soluble, but often is a good immunogen in its own right. For most purposes, either carrier will be adequate. Use whichever is more convenient.

Three other carriers that are used occasionally are ovalbumin, mouse serum albumin, or rabbit serum albumin. Ovalbumin can be used as a good carrier for most purposes. It is also a good choice for a second carrier when checking that antibodies are specific for the peptide itself and not the carrier. MSA or RSA may be used when the antibody response to the carrier molecule must be kept to a minimum.

BSA has 59 lysine (30–35 are available for coupling), 19 tyrosine, 35 cysteine, 39 aspartic acid, and 59 glutamic acid residues. Ovalbumin has 20 lysine, 10 tyrosine, 6 cysteine, 14 aspartic acid, and 33 glutamic acid residues.

AUSTRALIA

*Patents Act 1990*

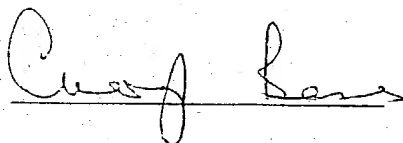
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-5  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001

A handwritten signature in cursive script, appearing to read 'C. J. Ross', written over a horizontal line.

(Signature of Witness)

Medical Practitioner